

Current approaches in the treatment of melanoma-II: Immuno- and gene therapy

KADRIYE ÇİFTÇİ, PETER TROVITCH

Temple University, School of Pharmacy, Philadelphia-U.S.A.

ABSTRACT

The incidence of melanoma has been increasing at a faster rate than that of any other solid tumor. Due to limited efficacy of chemotherapy and radiation therapy, immunotherapy has become a major focus of investigational treatment of distant and regionally metastatic melanoma. This review article focuses on the recent advances in immuno- and gene therapy approaches pursuing immunological strategies for the treatment of malignant melanoma. [Turk J Cancer 2003;33(3):127-136]

KEY WORDS:

Melanoma, skin cancer, chemotherapy, cytokines, interleukins, interferons, gene therapy, immunotherapy, viral vectors, non viral vectors, gene gun, liposomes

INTRODUCTION

The worldwide incidence of malignant melanoma is rising at a faster rate than any other cancer. Epidemiologic studies have shown that 10% of any population constitutes 80-90% of the malignant melanoma, and individuals with dysplastic nevi have a markedly increased risk for the development of disease including ocular melanoma (1). The social impact of malignant melanoma continues to warrant attention and the consistent failure of conventional chemotherapy and radiotherapy to alter the disease progression or survival makes the disease more problematic (2). Features of melanoma are immunologically provocative and they include: histopathology - lymphoid infiltration of dysplastic nevi and primary melanoma, disease course variability, paraneoplastic syndromes of depigmentation associated with prolonged survival, laboratory evidence of humoral and cellular immunity to autologous melanoma and a feature of historical responsiveness to crude immunopotentiators (3).

In recent years, immunotherapy has become a major focus of investigational treatment of distant and regionally metastatic melanoma as evidence has accumulated that immunostimulatory agents, interleukins (IL), interferons (IFN's), lymphokine-activated killer (LAK) cell infusion and tumor infiltrating lymphocyte infusion can cause tumor regression in 20% of patients with malignant melanoma (4-7). Among the cytokines, interferons (IFN's) can be immune enhancing and immunosuppressive with a maximal recorded overall response rate of 20-25% (4,5).

IFN- α potentiates NK cell cytotoxicity, macrophage activation and enhancement of MHC class I and II expression and has a direct antiproliferative effect. Although trials of IFN- γ have been disappointing to date, early results of IFN- γ gene modified autologous malignant melanoma cells hold promise (8).

Due to limited efficacy of chemotherapy of metastatic melanoma, there has been substantial interest in the use of adjuvant therapy in patients with a high risk of tumor recurrence. Several multi institutional studies have assessed the effect of post surgery interferon- α in high-risk melanoma patients (9). Adjuvant therapy of melanoma has been discussed in another manuscript from our laboratory (6).

The other cytokines, interleukins (IL's) have been shown to have varying roles ranging from down-regulating cell-mediated immunity and up-regulating antibody response in patients with metastatic melanoma. IL- α , TNF- α and IL-6 inhibit the proliferation and melanogenesis of normal human melanocytes in vitro. Normal human melanocytes express low levels of IL-10 mRNA, but do not produce detectable IL-10 levels (8,10). However, in halting the progression of effective treatment, toxicity related to non-specific immune activation limits the effectiveness of these immunotherapeutic approaches. The combination of chemotherapy plus immunotherapy appears to hold promise with high response rates and often-durable remissions reported, albeit at the expense of considerable treatment related toxicity (11).

Several investigators have shown that introducing genes such as IL-2, IL-4, IFN- γ , TNF- α , GM-CSF into tumor cells can lead to tumor regression in an immunocompetent host (12-15). Recombinant viruses, such as adenovirus, pox virus and vaccine viruses, which encode malignant melanoma tumor associated antigens (TAA), are being researched in immunization protocols for malignant melanoma (16). In addition, vaccine technology using plasmid DNA enables the presentation of large amounts of TAA on infected normal cells distant from the tumor and show great potential for the treatment of melanoma. The detail of this technology will be discussed in the next review article. One major advantage of this approach is it avoids certain forms of the toxicity associated with systemic use of viral vectors. However, overall immuno-gene therapy of melanoma is still in a highly experimental stage of development but may become safe and efficacious in the future.

Immunotherapy

Although advanced melanoma is relatively resistant to conventional therapy, several biologic response modifiers and cytotoxic agents have been reported to produce objective responses (3, 18-20). The two biologic therapies that appear most active against melanoma are interferons and interleukins.

Interferons

Interferons (IFN) are able to modulate host effector cell function, including the tumor cytolytic function of lymphocytes and monocytes. In addition, they have the capacity to regulate the distribution of circulating T-lymphocytes and the expression of tumor cell surface antigen, as well as class I and class II products of the major histocompatibility (MHC) locus (18).

In one study, low doses of IFN- α -2a, three times a week for 18 months, in high-risk melanoma patients was found to be safe and beneficial if started before clinically detectable node metastases developed. Out of the 489 patients studied there were 100 relapses and 59 deaths among the 244 IFN- α -2a treated patients compared with 119 relapses and 76 deaths among the 245 controls (19).

Pre-treatment of B16 melanoma cells with recombinant IFN- γ markedly increased their lung-colonizing capacity following i.v. injection into syngeneic mice as compared with control cells. This same treatment significantly increased resistance of B16 cells to splenic natural killer (NK) cells activity and also decreased sensitivity to NK cell mediated lysis. Moreover, IFN treatments altered Class I antigen expression causing dramatic increases in the expression of H-2Db antigen, in a pattern consistent with the possibility that increased H-2 antigen expression on B16 cells led to decreased NK cell sensitivity (21).

In another study, patients with malignant melanoma who are at high risk for recurrence after surgical resection have been treated with IFN- α -2b. It is hypothesized that IFN- α exerts a direct effect in the melanoma cell via the activation of signal transducer and activator of transcription (STAT) proteins. Cell lysates and patient tumor samples stimulated with IFN- α were incubated with radiolabeled oligonucleotides and then analyzed for STAT activation. Melanoma cell lines showed no evidence for constitutive STAT activation in the absence of cytokine stimulation but

exhibited rapid activation of STAT1 and STAT2 once treated with IFN- α . It was further found that the protein tyrosine kinase inhibitor, genistein, completely suppressed IFN- α STAT activation. Additionally, pre-treatments of melanoma tumor cells with IFN- γ resulted in a 4 log-fold decrease in the IFN concentration required for STAT activation and promoted the increase in expression of the STAT1 and STAT2 proteins (22).

IFN-stimulated gene factor 3 (ISGF3) mediates transcriptional activation of IFN-sensitive genes (ISGs). It has been established that the responsiveness of human melanoma cell lines to type-I IFNs correlated directly with their intracellular levels of ISGF3 components, particularly STAT 1. By treating IFN-resistant melanoma cell lines with IFN- γ before stimulation with type-I IFN, results showed an increase in levels of ISGF3 components and enhanced DNA-binding activation of ISGF3. This method of use of IFN- γ also enhanced the antiviral effect of IFN- β on the IFN-resistant melanoma cell line and also support a role of such IFN- γ priming in up-regulating ISGF3. This up-regulation would augment the responsiveness of IFN-resistant melanoma cell lines to type-I IFN and provide a molecular basis and justification for using sequential IFN therapy to enhance the use of IFNs in the treatment of melanoma (23).

Recent data demonstrated that the chemotherapy plus immunotherapy hold promise in the treatment of advanced melanoma. The combination of BCNU, cisplatin, dacarbazine (DTIC), interferon (IFN) and low dose tamoxifen was studied in 29 patients with metastatic melanoma. Five patients demonstrated complete remission and the median duration of response was 8 months. Generally, toxicity was manageable but myelosuppression, especially thrombocytopenia, was pronounced. Ten patients achieved partial remission for a median duration of 4 months (24).

A similar study was conducted in which a chemotherapy protocol was initiated first and then followed by surgical excision in patients with metastatic melanoma. All patients received cyclic chemotherapy, including one drug, or a combination of DTIC, BCNU, cisplatin, α -IFN, and tamoxifen. If after two cycles the tumor showed no response, the metastases were surgically resected. However, if there was a response, chemotherapy continued until maximum response was obtained, followed by resection of the site even if no tumor could be clinically detected. The purpose of the chemotherapy was not to decrease the original tumor

size and reduce the surgical resection, but to eradicate the undetectable tumor cells elsewhere in the body that the resection would not incorporate. It was found that there was a considerable improvement in the median survival of the patients undergoing resection (35 months) compared to that of the patients who refused surgical resection following chemotherapy (11.5 months); thus leading to suggest that surgical resection treated in combination with chemotherapy may significantly improve survival rate (11).

It has also been reported that combined chemotherapeutic immunotherapy with cyclophosphamide (CY), thymosin- α 1 (T α 1) and low dose interferon- $\alpha\beta$ (IFN $\alpha\beta$) has significant anti-tumor effects in mouse B16 melanoma. It has been tested whether increasing the dose of T α 1 could increase the anti-tumor activity of triple combination chemotherapeutic immunotherapy. Results revealed that chemotherapeutic immunotherapy with high dose T α 1 caused complete tumor regression for 27.5 days after tumor cell injection (3.9 times longer than untreated controls) and delayed tumor relapse. The protocol also significantly increased the median survival time of treated mice, and cured an average of 23% of animals (25).

Interleukins (IL's)

The role of soluble mediators (IL's), 2, 4, 7, 10 and 12 in antitumor immunity via activation of T-cells was evaluated in patients with metastatic melanoma. Combination trials of IL-2 and IL-4 have shown no increase in responsiveness of melanoma or other tumors when compared to IL-2 alone (20). However, enhanced expansion of tumor-infiltrating lymphocytes has been observed in a low dose combination of IL-2 and IL-4. IL-7 induces proliferation and lymphokine-activated killer (LAK) cell activity and also increases the proliferation of murine B and T cells located in the spleen and lymph nodes. IL-10 was originally defined as a factor inhibiting IL-2 and γ -interferon production but has been found to synergize with IL-2, IL-4, and IL-7 in the growth of thymic and peripheral T cells. IL-12, a heterodimeric cytokine, enhances proliferation and cytolytic capacity of T cells and large granular lymphocytes. IL-12 also synergizes with IL-2 in the induction of LAK cells, and induces the secretion of interferon- γ and tumor necrosis factor- α (20).

Using genetically engineered fibroblasts in murine tumor models the effects of panacrine secretion of IL-12 on tumor establishment and vaccination models were examined. Effectiveness in this system was related to the

amount of IL-12 expression and significant delay of establishment of tumor was noted with relatively small amount of IL-12 secretion. Larger amounts of secreted IL-12 provided no additional therapeutic benefit. Local delivery of IL-12 inhibited tumor growth in a dose dependent manner but led to the development of an antitumor immune response when IL-12 is expressed at the tumor site at relatively small amounts.

These results suggest that IL-12, like IL-2, -4, -6, -7 and granulocyte-macrophage colony-stimulating factor can induce an immune response against poorly immunogenic tumors (26). IL-12 was also found to bind to a receptor on T-cells and natural killer (NK) cells, promoting the induction of primarily a TH1 response *in vitro* and *in vivo*. These activities suggest that IL-12 alone, or in combination with IL-2, might have antitumor effects. Furthermore, Tahara et al. (26) reported that panacrine secretion of IL-12 delayed tumor formation and promoted antitumor immunity. In a similar study, the *i.p.* injection of IL-12 was found to significantly suppress the growth of inoculated (*s.c.*) B16 melanoma for up to 2 weeks after the last injection of IL-12. The *in vivo* depletion of either CD4(+) or CD8(+) T cells abrogated the antitumor activity of IL-12 and diminished the apparent autocrine stimulation of IL-12 release. The antitumor activity observed after IL-12 treatment was diminished by the *in vivo* administration of either anti-IL-12 or anti-CD40L monoclonal antibodies. These results suggest that the endogenous production of IL-12 resulting from the CD40-CD40L interaction between antigen-presenting cells and CD4(+) T cells in the tumor-draining lymph nodes may play a role in the persistence of the antitumor effects (27).

The tumor-binding and lymphocyte-activating capability of a recombinant fusion protein consisting of a tumor-selective human/mouse chimeric anti-ganglioside GD2 antibody and recombinant human IL-2 was investigated by Hank et al. (28). The fusion protein was bound specifically to GD2-positive melanoma and neuroblastoma tumor cell lines, and its IL-2 component stimulated *in vitro* proliferation of an IL-2 dependent cell line. The IL-2 presented by the fusion protein, when bound to tumor cells, induced proliferation of IL-2 responsive cells suggesting that localization of IL-2 at the site of contact between tumor and effector cells is an effective way of representing this cytokine to IL-2 responsive cells (28).

Gp130 acts as a common transducing signal chain for all receptors belonging to the IL-6 receptor family. The IL-6 cytokines often modulate tumor phenotype and control the proliferation of many cell lines. Melanoma cell lines release, *in vitro* and *in vivo*, soluble gp130, a potential antagonist of cytokines from the IL-6 family (29). Although early stage lesions were growth inhibited by exogenous IL-6 *in vivo*, cell lines from advanced-stage lesions were resistant to such growth inhibition. Moreover, endogenous IL-6 can indeed function as a growth stimulator for human cutaneous melanomas *in vivo* (30). The time course or endogenous IL-6 secretion was studied in patients treated with cisplatin, IL-2, and IFN- α to evaluate whether serum IL-6 can be useful as a disease marker in metastatic malignant melanoma. The relationship of endogenous IL-6 concentrations to the tumor burden was also evaluated. The IL-6 levels were higher in patients with high tumor burden than in patients with low tumor burden and a higher serum IL-6 level was observed in nonresponding as compared to responding patients and remained higher regardless of tumor burden. Endogenous IL-6 may play a role in the failure of IL-2 therapy in such patients, since the very early IL-6 increase is correlated with tumor mass and nonresponse to biochemotherapy (31).

Peripheral blood mononuclear cells (PBMCs) of patients newly diagnosed with metastatic melanoma were incubated with different doses of recombinant IL-15 and tested against autologous tumor cells, LAK sensitive cell lines, as well as NK sensitive cell lines. The effect of IL-15 was found to be both time and dose dependent, with peak activity detected after 2 or 3 days of culture. Incubation of patients' PBMCs with IL-15 for 6 hours resulted in the up-regulation of perforin mRNA transcription. These findings suggest that LAK activity can be generated from melanoma patients' PBMCs in the presence of IL-15 to lyse autologous tumor cells in a non-MHC restricted manner (32).

The bacterial superantigen staphylococcal enterotoxin A (SEA) is a potent inducer of CTL (cytotoxic T-lymphocytes) activity and was genetically fused to a Fab fragment of the C215 tumor-reactive antibody (33). Strong reduction of lung metastasis was seen in mice carrying established lung metastasis but important anti-tumor effector functions, such as IFN- γ secretion and CTL activity, gradually declined during therapy. It was shown that Fab-SEA immunotherapy was strongly potentiated by Fab-IL-2 co-administration and this combined therapy prolonged the

immune response *in vivo*, limited the development of immunological unresponsiveness and promoted maximal anti-tumor effects. The immune response after combination therapy was characterized by substantially augmented IFN- γ and TNF- α production and strong CTL activity. This combination therapy, in animals carrying the highly aggressive B16 melanoma, resulted in a complete cure in 90% of tumor-bearing animals, whereas only 10% long-term survival was seen in Fab-SEA or Fab-IL-2 treated animals (33).

IL-8 may serve as the angiogenic factor distinguishing benign from malignant cells. Expression of IL-8 by human melanoma cells correlates with their metastatic potential *in vivo* (34). Moreover, UVB (Ultraviolet B) irradiation of primary cutaneous melanoma induces IL-8 mRNA and protein production and increases both tumor growth and metastasis in nude mice. Transfection of nonmetastatic and IL-8 negative melanoma cells with the IL-8 gene rendered them highly tumorigenic and increased their metastatic potential in mice. The IL-8 transfected cells displayed upregulation of MMP-2 (a type IV collagenase) expression activity along with increased invasiveness. Activation of MMP-2 by IL-8 can enhance the invasion of host stroma by tumor cells and increases angiogenesis, and hence, metastasis. In addition to UVB, IL-8 can also be upregulated by hypoxia conditions (35).

IL-10 has the physiological role of down-regulating cell-mediated immunity and is present in most metastatic melanoma tissues. The purpose of Sato et al's experiment (35) was to determine whether melanoma metastases produce IL-10 protein. Out of 35 melanoma tissue samples, 30 produced IL-10 after 24-hour incubation. After 7 or 14 days in tissue culture, melanoma cells continued to produce IL-10 but only at about 10% of the levels of freshly dissociated tissues. Moreover, of eight melanoma cell lines established from these cultures, only one produced IL-10 protein. IL-10 production was increased by depletion of leukocytes, suggesting that the primary source was the melanoma cells. Finally, 10 of 55 patients with clinically evident metastases showed elevations of circulating IL-10; three patients who had been melanoma-free developed high serum IL-10 levels, concurrent with the appearance of distant metastases. The data indicated that production of IL-10 is characteristic of metastatic melanomas and raised the possibility that this cytokine allows tumors to avoid or to modulate immunological attack (10). In another study,

the influence of IL-10 on a human melanoma cell line, A375P, tumor growth and metastatic properties was investigated. A276P-IL-10 cells produced significantly slower s.c. tumors and fewer lung metastases than control cells. Also, the tumorigenicity of the human melanoma A375SM and the murine melanoma B16-BL6 cells were also significantly inhibited when they were admixed with A37P-IL-10 before s.c. injection into mice. It was concluded that the production of IL-10 by tumor cells inhibits macrophage-derived angiogenic factors, and hence, tumor growth and metastasis (36). It also has been shown that IL-10-pretreatment of dendritic cells not only reduces their allostimulatory capacity, but also induces an antigen-specific energy in cytotoxic CD8(+) T cells, a process that might be a mechanism of tumors to inhibit immune surveillance by converting dendritic cells into tolerogenic antigen-presenting cell (37). In a similar study, it was found that cellular IL-10 (cIL-10) has both stimulatory and inhibitory effects on diverse cell types and was administered systemically to mice bearing established melanoma. At high doses cIL-10 induced rejection of tumors, delayed tumor outgrowth or resulted in complete cure. Sublethal irradiation of mice prior to tumor inoculation abrogated the IL-10 effect. Cured mice were immune to subsequent rechallenge with 10-fold higher inoculation with the same tumor (38).

Gene therapy

The concept that gene transfer might be applicable in treating diseases, such as cancer, is founded on the extraordinary advances of the past two decades in the area of recombinant DNA technology. Among the other gene therapy approaches, the transfer of genes for cytokines or the other immunomodulatory products delivered to cancer cells stimulate immune recognition of these cells (12-14).

Artificially engineered viruses are the most efficient means of gene transfer among the expression systems. Usually therapeutic genes are inserted at the expense of the viral genetic information. Thus, once the gene has been delivered the virus can neither replicate nor harm the patient. Rarely, replication defective viral vectors may be replication-competent that poses a risk to the patient. There are several types of viruses currently used as vector systems, these include adenoviruses, adenoassociated virus (AAV) and retroviruses (39-42).

Retroviral vectors are the most frequently used and persist in the target cells over long period of time because they can be integrated in the host genome (42). Similarly, AAV vectors become integrated into the host genome, whereas adenoviral vector endure as unintegrated DNA for approximately two weeks (42). The incorporation of large DNA fragments into viral vectors requires the deletion of wild type viral DNA sequences (i.e., E1, E2 and E4) and deletion results in a virus deficient vector (39,41,42). Their limited infection spectrum and a weak or lacking immune response in the host further distinguishes retroviral vectors. In contrast, an undesired immune response is a familiar drawback of a repeated delivery of AV (39).

Viral vectors

Transfection of human or murine melanoma cells with the co-stimulatory B7-1 molecule induces effective antitumor response. A study was done to evaluate the in vitro and in vivo immune response associated with this AV transduction in these melanoma cells. Results revealed that this AV transduction of human melanoma cells with B7-1 leads to high-level transgene expression in vitro and in vivo and did not affect MHC class 1 and 2 expression. By contrast, tumoral injection of an AV encoding murine B7-1 failed to eliminate established murine melanoma despite high level transgene expression in tumor cells (40).

Genes encoding melanoma associated antigens MART-1 or GP100 were inserted into an AV and administered into 54 patients either alone or followed by administration of IL-2. Only 1 patient (of 16) receiving the recombinant AV-MART-1 experienced a complete response however, all other patients showed objective responses. It has been concluded that high levels of neutralizing antibody present in the patients' sera prior to the treatment may have impairment in the ability of the virus to immunize patient (41).

Melanoma cells are normally resistant to TNF mediated killing. It has been shown that transcription factors of the NF- κ B family, which themselves are activated by TNF, could protect cells against apoptotic cell death. A recombinant AV was generated expressing a dominant mutant form of I κ B α , under the control of a CMV promoter. It was shown that AV mediated inhibition of NF- κ B function rendered melanoma cells susceptible to the cytotoxic effects of TNF (43).

For retroviral vectors (RV), a study was done to determine the safety of treating melanoma patients with RV mediated IFN- γ gene transduced autologous tumor cells. Irradiated, transduced melanoma cells expressing the IFN- γ gene were injected s.c. every two weeks with increasing doses for 6 injections. Five patients received injection and there were no toxicity attributed to the RV. One patient remained disease free after 13-injections that followed removal of metastases. The overall results revealed that injections of autologous tumor cells transduced by IFN- γ gene were well tolerated but the ability to develop primary autologous melanoma cell lines was limited (42).

The behavior of IFN- α 1 transfected B16 melanoma cells in vitro and their growth in vivo in synergetic and allogenic mice were characterized. Results showed that IFN- α 1 gene transfection alters the phenotype of these cells in such a way that they are totally rejected by allogenic mice and have reduced tumorigenicity in synergetic mice. Moreover, IFN- α 1, transfected cells can induce immunity in allergenic mice (44).

In most current cancer gene therapy, tumor cells were removed by biopsy and genetically modified ex vivo before being returned to the patient. This method is slow, costly and is limited to that patient by which a biopsy can be readily taken. For cancer gene therapy to have widespread clinical application, it will be necessary to avoid ex vivo gene transfer. This approach was taken in a study to generate RV that specifically infects melanoma cells. Two strategies were compared. First, was to extend the tropism of an ecotropic envelope to human cells. Second, was to enhance tropism of an amphotropic envelope for melanoma cells. It was found that chimeric RV envelopes, incorporating a single chain antibody (ScFv) directed against high molecular weight melanoma associated antigen (HMWMAA) at the immune terminus are correctly processed and incorporated into virions. ScFv ecotropic envelope chimerase allow specific but low titer, targeting of HMWMAA (+) cells, when co-expressed with ecotropic envelopes. ScFv amphoteric envelope chimerase bind specifically to HMWMAA positive cells and allow preferential infection at high titer (45)

Non viral vectors

There are several non-viral methods of gene transfer that pose less potential risk to the patient, particularly in terms of harmful integration into the host genome or

potential infection (17). This includes the use of mammalian expression vectors containing certain regulatory (e.g. promoter) and selection elements in addition to the therapeutic gene. Such transfer techniques include calcium phosphate precipitation, DEAE-Dextran transfection, electroporation, in vitro DNA microinjection; receptor mediated DNA transfection, liposomal DNA complexes, direct DNA injection in vivo and ballistic gene transfer. Frequently these are not useful clinically, due to low transfection efficiency or technical limitations. However, two gene transfer techniques are increasingly employed: 1.) The direct injection, "naked DNA", resulting in the expression of the encoded proteins. This in vivo approach can be used for transient production of various therapeutic proteins including infectious or tumor antigens, 2.) Naked DNA coated on gold microparticles can be directly propelled into in vivo target cells and this process is known as "gene gun" (17,46).

The gene gun technology is especially useful for transfection of tissue ex-plants, cell clumps, organoids and tumor cells as freshly isolated cell aggregates or as the primary cell cultures. In addition, various leukocytes, including peripheral blood lymphocytes, splenocytes, macrophages, T-cells and CD34+ cells can be effectively transfected for transient gene expression using the gene gun technique (46-48). This technique will be discussed in detail later in this article.

Liposomes: Tumor cells require large amounts of cholesterol for the synthesis of membrane due to their rapid proliferation. Therefore, many tumors express relatively high levels of low-density lipoprotein (LDL) receptors on their membranes and therefore are an attractive target to deliver drug therapy to the tumor cells. One study examined the effect of the small apolipoprotein E (APOE) containing liposomes on cultured B16 melanoma cells. Cross competition studies indicated that APO-E liposomes are bound by the LDL receptor. APOE is found to be essential for the LDL receptor recognition because liposomes lacking APO-E were 20-50 fold less effective than APO-E containing liposomes. The tumor localizing properties of APO-E liposomes and the disposition of an incorporated lipophilic derivative of Daunorubicin (LAD) was also investigated in B16 tumor bearing mice. LAD loaded APO-E liposomes were taken up and processed by the major LDL receptor expressing organs. Of all other tissues, tumor shows the highest uptake. The disposition of LAD followed the pattern

of liposomal carrier. Thus, this constitutes an attractive novel option for melanoma therapy (49).

It is beneficial to determine recombinant gene expression and investigate the safety and potential toxicity of this therapy. It has been studied that the gene encoding a foreign major histocompatibility complex protein, HLA-B7, was introduced into HLA-B7 negative patients with advanced melanoma by injection of DNA liposome complexes in an effort to demonstrate gene transfer. Six courses of treatment were completed in five patients with stage-IV melanoma. Plasmid DNA was detected within biopsies of treated tumor nodules three to seven days after injection but was not found in the serum at any time. Recombinant HLA-B7 protein was demonstrated in tumor biopsy tissue in all five patients, and immune responses to HLA-B7 and autologous tumor could be detected. No antibodies to DNA were detected in any patient. One patient demonstrated regression of injected nodules on two independent treatments, which was accompanied by regression at distance site (50).

The virus free transfer of a "suicide gene", herpes simplex virus thymidine kinase (HsV-Tk) from a plasmid into tumor cells can be done both in vitro and in vivo. This plasmid was used both alone and in liposomes to transduce B-16 melanoma cells. In vitro, a 5-day treatment with ganciclovir after transfection with the HsV-Tk gene and liposomes induced significant lysis of B-16 melanoma cells. The efficacy of transfection was determined using liposomes harboring β -galactosidase reporter gene and was found to be around 10%. The cytotoxicity observed resulted presumably from a large bystander effect. In vivo, direct transfer of the Tk-DNA into established B-16 melanoma tumors followed by i.p. ganciclovir treatment induced a 50% reduction of tumor weight after and an increased necrosis. No necrosis was detected in normal tissue surrounding the tumor or elsewhere (51). A similar study was done in mice bearing B-16 F1 melanoma tumors. On days 11 and 14 an intratumoral injection of either naked plasmid counting the HsV-Tk gene or pAGO lipofectamine complexes was given. Ganciclovir was given for 5 days starting on day 14. Tumor weight reduction was observed at 40-50% versus controls and the analysis of tumors showed large areas of necrosis (85%) (52).

Vile et al. (53) used the murine tyrosinase gene to direct expression of the HSV-Tk gene specifically to murine cells, while not permitting expression in a range of other cells. Expression of the HSV-Tk gene in melanoma cells rendered

them sensitive to killing ganciclovir. There was also a substantial bystander killing effect when expressed cells were mixed with non-transfected parental B-16 cells. Direct injection of the Tk-gene under control of the tyrosinase promoter into established tumors in mice, followed by treatment of ganciclovir, led to significant reduction in resulted tumor size. There was a 100% cell death of the Tk-expression B-16 clone after 12 days in culture at 1 μ m/ml ganciclovir concentration (53).

Gene Gun: The use of granulocyte macrophage colony stimulating factor (GMCSF) in the treatment of a tumor appears to be the present "gene of choice". The first study believed that GMCSF cDNA in a plasmid expression vector could be effective if introduced into resting tumor cells, and that efficient expression of transgenic GMCSF by the transfected tumor cells would confer an effective immune response against tumor. GMCSF cDNA expression vectors were coated onto gold particles and accelerated with a gene gun device into mouse and human tumor cells. Human tumor cells transfected within 4 h of surgery produced significant levels of transgenic human GMCSF protein in vitro. The antitumor efficacy of this non-viral approach was tested using irradiated B-16 tumor cells then was transfected with mGMCSF cDNA and injected into mice. Subsequent challenge of these mice with non-irradiated non-transfected B-16 tumor cells showed that 58% of the animals were protected from the tumor. In contrast only 2% of the control animals were protected by prior treatment (54).

In another study, GMCSF cDNA in a non viral expression vector was inserted into M21 cells, a human melanoma and B-16, in murine melanoma, cells by particle mediated gene transfer. The ability of transfected tumor cells to generate tumor specific immune response was evaluated in an in vitro mixed lymphocyte tumor cells assay and in an in vivo murine tumor protection model. Peripheral blood lymphocytes (PBL's) co-cultured with human GMCSF transfected tumor cells were 3-5 fold more effective at lysis of the parenteral tumor cells than were PBL's incubated with irradiated tumor cells and exogenous human GMCSF. Mice immunized with murine GMCSF transfected irradiated B-16 murine melanoma cells were protected from subse-

quent tumor challenge, whereas mice immunize with the non transfected tumors and cutaneous transfection of murine GMCSF cDNA at the vaccination side developed tumors more frequently. This indicates that GMCSF protein expressed human and murine tumor cells is a superior antitumor immune stimulant compared with exogenous GMCSF in the tumor microenvironment (15). Lastly, this non-viral approach against melanoma was extended by using the poorly immunogenic murine myeloma MPC-11 model.

Vaccination with the transfected GMCSF expressing MPC-11 cells induced a potent antitumor CTL response. Furthermore nearly 100% of the tumor free mice were able to reject a tumor rechallenge. A number of tested human primary tumors including myeloma cells have failed to produce high level of GMCSF after gene gun transfection. To circumvent this low transfection efficiency, they showed that combining irradiated tumor cells to present tumor antigens together with gene gun transfected fibroblast to provide GMCSF induced effective tumor rejection. These results suggested combining irradiated tumor cells with gene gun transfected fibroblasts revealed an antitumor response and may allow for a wider application of this approach to cancer immunotherapy (55).

CONCLUSION

In summary, improved diagnosis and treatment of malignant melanoma have led to high survival rates despite the unchanged character of poor prognosis of advanced disease. While surgical excision is an effective treatment for patients with early and localized disease, it is not considered a useful intervention for patients with advanced melanoma. Some chemotherapy approaches for melanoma achieve response rates of ~ 50% but reported survival rate data clearly demonstrates the need for improved treatment approaches. Immunotherapy is already established as an available method in treating the advanced melanoma however its limitations and potential problems should always be under consideration. In addition, gene therapy appears to be a promising approach for the treatment of melanoma but the ongoing clinical studies still focus on safety and tolerability rather than efficacy.

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